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(57) Abstract

The invention relates to relatively short peptides (termed ap-conotoxins herein), about 10-20 residues in length, which are naturally available in minute amounts in the venom of cone snails or analogous to the naturally available peptides, and which preferably include two disulfide bonds. These conotoxin peptides have analgesic activity and are thus useful for treating or preventing pain.

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TITLE OF THE INVENTION

CONOTOXIN PEPTIDES

This invention was made with Government support under Grant No. PO1 GM48677 awarded by the National Institute of General Medical Sciences, National Institutes of Health, Bethesda, Maryland. The United States Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

The invention relates to relatively short peptides (termed ap-conotoxins herein), about 10-20 residues in length, which are naturally available in minute amounts in the venom of the cone snails or analogous to the naturally available peptides, and which preferably include two disulfide bonds. These conotoxin peptides have analgesic activity and are thus useful for treating or preventing pain.

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference, and for convenience are referenced in the following text by author and date and are listed alphabetically by author in the appended bibliography.

The predatory cone snails (*Conus*) have developed a unique biological strategy. Their venom contains relatively small peptides that are targeted to various neuromuscular receptors and may be equivalent in their pharmacological diversity to the alkaloids of plants or secondary metabolites of microorganisms. Many of these peptides are among the smallest nucleic acid-encoded translation products having defined conformations, and as such, they are somewhat unusual. Peptides in this size range normally equilibrate among many conformations. Proteins having a fixed conformation are generally much larger.

The cone snails that produce these peptides are a large genus of venomous gastropods comprising approximately 500 species. All cone snail species are predators that inject venom to capture prey, and the spectrum of animals that the genus as a whole can envenomate is broad. A wide variety of hunting strategies are used, however, every *Conus* species uses fundamentally the same basic pattern of envenomation.

Several peptides isolated from *Conus* venoms have been characterized. These include the α-, μ- and ω-conotoxins which target nicotinic acetylcholine receptors, muscle sodium channels, and neuronal calcium channels, respectively (Olivera et al., 1985). Conopressins, which are vasopressin analogs, have also been identified (Cruz et al.. 1987). In addition, peptides named conantokins have been isolated from *Conus geographus* and *Conus tulipa* (Mena et al., 1990; Haack et al., 1990). These peptides have unusual age-dependent physiological effects: they induce a sleep-like state in mice younger than two weeks and hyperactive behavior in mice older than 3 weeks (Haack et al., 1990). The isolation, structure and activity of κ-conotoxins are described in U.S. Patent No. 5,633,347. Recently, peptides named contryphans containing D-tryptophan residues have been isolated from *Conus radiatus* (U.S. Serial No. 09/061,026), and bromo-tryptophan conopeptides have been isolated from *Conus imperialis* and *Conus radiatus* (U.S. Serial No. 08/785,534).

It is desired to identify additional conopeptides having activities of the above conopeptides, as well as conotoxin peptides having additional activities.

SUMMARY OF THE INVENTION

The invention relates to relatively short peptides (termed ap-conotoxins herein), about 10-20 residues in length, which are naturally available in minute amounts in the venom of the cone snails or analogous to the naturally available peptides, and which preferably include two disulfide bonds. These conotoxin peptides have analgesic activity and are thus useful for treating or preventing pain.

More specifically, the present invention is directed to ap-conotoxin peptides having the general formula I:

Xaa_1-Gly-Xaa_2-Cys-Cys-Gly-Xaa_3-Xaa_4-Xaa_5-Cys-Xaa_6-Xaa_7-Cys (SEQ ID NO:1), wherein Xaa_1 is Asn or des-Xaa_1; Xaa_2 is Val or Ile; Xaa_3 is Val, Tyr, mono-halo-Tyr, di-halo-Tyr, O-sulpho-Tyr, O-phospho-Tyr, nitro-Tyr; Xaa_4 is Ser, Thr, Lys, N-methy-Lys, N,N-dimethyl-Lys, N,N,N-trimethyl-Lys, Arg, homoarginine or ornithine; Xaa_5 is Leu or Phe; Xaa_6 is His, halo-His, Tyr, mono-halo-Tyr, di-halo-Tyr, O-sulpho-Tyr, O-phospho-Tyr, nitro-Tyr; and Xaa_7 is Pro or hyroxy-Pro. The C-terminus may contain a free carboxyl group or an amide group. The halo is preferably chlorine or iodine, more preferably iodine.

The present invention is also directed to novel specific ap-conotoxin peptides of general formula I having the formulas:

Asn-Gly-Val-Cys-Cys-Gly-Xaa_1-Xaa_2-Leu-Cys-His-Xaa_3-Cys (SEQ ID NO:2);

Gly-Val-Cys-Cys-Gly-Xaa_1-Xaa_2-Leu-Cys-His-Xaa_3-Cys (SEQ ID NO:3) and

Gly-Ile-Cys-Cys-Gly-Val-Ser-Phe-Cys-Xaa_1-Xaa_3-Cys (SEQ ID NO:4),

wherein Xaa_1 is Tyr, mono-halo-Tyr, di-halo-Tyr, O-sulpho-Tyr, O-phospho-Tyr, nitro-Tyr; Xaa_2 is Lys, N-methy-Lys, N,N-dimethyl-Lys or N,N,N-trimethyl-Lys; Xaa_3 is Pro or hydroxy-Pro, preferably hydroxy-Pro; and the C-terminus contains a carboxyl or amide group. The halo is preferably chlorine or iodine, more preferably iodine.

More specifically, the present invention is directed to the following ap-conotoxin peptides of general formula I:

Mar1: SEQ ID NO:2, wherein Xaa_1 is Tyr; Xaa_2 is Lys and Xaa_3 is hydroxy-Pro;

Mar2: SEQ ID NO:3, wherein Xaa_1 is Tyr; Xaa_2 is Lys and Xaa_3 is hydroxy-Pro; and

U036: SEQ ID NO:4, wherein Xaa_1 is Tyr and Xaa_3 is hydroxy-Pro.

The present invention is further directed to the identification of the nucleic acid sequences encoding these peptides and their propeptides and the identication of nucleic acid sequence of additional ap-conotoxin peptides.

SUMMARY OF THE SEQUENCE LISTING

SEQ ID NO:1 is generic formula I for ap-conotoxin peptides. SEQ ID NO:2 is a generic formula for the peptide Mar1. SEQ ID NO:3 is a generic formula for the peptide Mar2. SEQ ID NO:4 is a generic formula for the peptide U036. SEQ ID NO:5 is the nucleotide sequence of a degenerate primer for 3' RACE of the Mar1 gene. SEQ ID NO:6 is the nucleotide sequence of a degenerate primer for 5' RACE of the Mar 1 gene. SEQ ID NO:7 is the nucleotide sequence of a universal amplification primer. SEQ ID NO:8 is a nucleotide sequence for the gene coding for the Mar1 propeptide. SEQ ID NO:9 is an amino acid sequence of the Mar1 propeptide.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The invention relates to relatively short peptides (termed ap-conotoxins herein), about 10-20 residues in length, which are naturally available in minute amounts in the venom of the cone snails or analogous to the naturally available peptides, and which preferably include two disulfide bonds. These conotoxin peptides have analgesic activity and are thus useful for treating or preventing pain.

The present invention, in another aspect, relates to a pharmaceutical composition comprising an effective amount of an ap-conotoxin peptide. Such a pharmaceutical composition has the capability of acting as analgesic agents.

The ap-conotoxin peptides described herein are sufficiently small to be chemically synthesized. General chemical syntheses for preparing the foregoing α-conotoxin peptides are described hereinafter. Various ones of the ap-conotoxin peptides can also be obtained by isolation and purification from specific *Conus* species using the technique described in U.S. Patent No. 4,447,356 (Olivera et al., 1984), the disclosure of which is incorporated herein by reference.

Although the ap-conotoxin peptides of the present invention can be obtained by purification from cone snails, because the amounts of ap-conotoxin peptides obtainable from individual snails are very small, the desired substantially pure ap-conotoxin peptides are best practically obtained in commercially valuable amounts by chemical synthesis using solid-phase strategy. For example, the yield from a single cone snail may be about 10 micrograms or less of ap-conotoxin peptide. By "substantially pure" is meant that the peptide is present in the substantial absence of other biological molecules of the same type; it is preferably present in an amount of at least about 85% purity and preferably at least about 95% purity. Chemical synthesis of biologically active ap-conotoxin peptides depends of course upon correct determination of the amino acid sequence.

The ap-conotoxin peptides can also be produced by recombinant DNA techniques well known in the art. Such techniques are described by Sambrook et al. (1989). The peptides produced in this manner are isolated, reduced if necessary, and oxidized to form the correct disulfide bonds.

One method of forming disulfide bonds in the peptides of the present invention is the air oxidation of the linear peptides for prolonged periods under cold room temperatures or at room temperature. This procedure results in the creation of a substantial amount of the bioactive, disulfide-linked peptides. The oxidized peptides are fractionated using reverse-phase high performance liquid chromatography (HPLC) or the like, to separate peptides having different linked configurations. Thereafter, either by comparing these fractions with the elution of the native material or by using a simple assay, the particular fraction having the correct linkage for maximum biological potency is easily determined. However, because of the dilution resulting from the presence of other fractions of less biopotency, a somewhat higher dosage may be required.

The peptides are synthesized by a suitable method, such as by exclusively solid-phase techniques, by partial solid-phase techniques, by fragment condensation or by classical solution couplings.

In conventional solution phase peptide synthesis, the peptide chain can be prepared by a series of coupling reactions in which constituent amino acids are added to the growing peptide chain in the desired sequence. Use of various coupling reagents, e.g., dicyclohexylcarbodiimide or diisopropylcarbonyldimidazole, various active esters, e.g., esters of N-hydroxyphthalimide or N-hydroxy-succinimide, and the various cleavage reagents, to carry out reaction in solution, with subsequent isolation and purification of intermediates, is well known classical peptide methodology. Classical solution synthesis is described in detail in the treatise, "Methoden der Organischen Chemie (Houben-Weyl): Synthese von Peptiden," (1974). Techniques of exclusively solid-phase synthesis are set forth in the textbook, "Solid-Phase Peptide Synthesis," (Stewart and Young, 1969), and are exemplified by the disclosure of U.S. Patent 4,105,603 (Vale et al., 1978). The fragment condensation method of synthesis is exemplified in U.S. Patent 3,972,859 (1976). Other available syntheses are exemplified by U.S. Patents No. 3,842,067 (1974) and 3,862,925 (1975). The synthesis of peptides containing γ-carboxyglutamic acid residues is exemplified by Rivier et al. (1987), Nishiuchi et al. (1993) and Zhou et al. (1996).

Common to such chemical syntheses is the protection of the labile side chain groups of the various amino acid moieties with suitable protecting groups which will prevent a chemical reaction from occurring at that site until the group is ultimately removed. Usually also common is the protection of an α-amino group on an amino acid or a fragment while that entity reacts at the carboxyl group, followed by the selective removal of the α-amino protecting group to allow subsequent reaction to take place at that location. Accordingly, it is common that, as a step in such a synthesis, an intermediate compound is produced which includes each of the amino acid residues located in its desired sequence in the peptide chain with appropriate side-chain protecting groups linked to various ones of the residues having labile side chains.

As far as the selection of a side chain amino protecting group is concerned, generally one is chosen which is not removed during deprotection of the α-amino groups during the synthesis. However, for some amino acids, e.g., His, protection is not generally necessary. In selecting a particular side chain protecting group to be used in the synthesis of the peptides, the following general rules are followed: (a) the protecting group preferably retains its protecting properties and is not split off under coupling conditions, (b) the protecting group should be stable under the reaction conditions selected for removing the α-amino protecting group at each step of the synthesis, and (c) the side chain protecting group must be removable, upon the completion of the synthesis

containing the desired amino acid sequence, under reaction conditions that will not undesirably alter the peptide chain.

It should be possible to prepare many, or even all, of these peptides using recombinant DNA technology. However, when peptides are not so prepared, they are preferably prepared using the Merrifield solid-phase synthesis, although other equivalent chemical syntheses known in the art can also be used as previously mentioned. Solid-phase synthesis is commenced from the C-terminus of the peptide by coupling a protected α-amino acid to a suitable resin. Such a starting material can be prepared by attaching an α-amino-protected amino acid by an ester linkage to a chloromethylated resin or a hydroxymethyl resin, or by an amide bond to a benzhydrylamine (BHA) resin or para-methylbenzhydrylamine (MBHA) resin. Preparation of the hydroxymethyl resin is described by Bodansky et al. (1966). Chloromethylated resins are commercially available from Bio Rad Laboratories (Richmond, CA) and from Lab. Systems, Inc. The preparation of such a resin is described by Stewart and Young (1969). BHA and MBHA resin supports are commercially available, and are generally used when the desired polypeptide being synthesized has an unsubstituted amide at the C-terminus. Thus, solid resin supports may be any of those known in the art, such as one having the formulae -O-CH_2-resin support, -NH BHA resin support, or -NH-MBHA resin support. When the unsubstituted amide is desired, use of a BHA or MBHA resin is preferred, because cleavage directly gives the amide. In case the N-methyl amide is desired, it can be generated from an N-methyl BHA resin. Should other substituted amides be desired, the teaching of U.S. Patent No. 4,569,967 (Kornreich et al., 1986) can be used, or should still other groups than the free acid be desired at the C-terminus, it may be preferable to synthesize the peptide using classical methods as set forth in the Houben-Weyl text (1974).

The C-terminal amino acid, protected by Boc or Fmoc and by a side-chain protecting group, if appropriate, can be first coupled to a chloromethylated resin according to the procedure set forth in K. Horiki et al. (1978), using KF in DMF at about 60°C for 24 hours with stirring, when a peptide having free acid at the C-terminus is to be synthesized. Following the coupling of the BOC-protected amino acid to the resin support, the α-amino protecting group is removed, as by using trifluoroacetic acid (TFA) in methylene chloride or TFA alone. The deprotection is carried out at a temperature between about 0°C and room temperature. Other standard cleaving reagents, such as HCl in dioxane, and conditions for removal of specific α-amino protecting groups may be used as described in Schroder & Lubke (1965).

After removal of the α-amino-protecting group, the remaining α-amino- and side chain-protected amino acids are coupled step-wise in the desired order to obtain the intermediate compound defined hereinbefore, or as an alternative to adding each amino acid separately in the synthesis, some of them may be coupled to one another prior to addition to the solid phase reactor. Selection of an appropriate coupling reagent is within the skill of the art. Particularly suitable as a coupling reagent is N,N'-dicyclohexylcarbodiimide (DCC, DIC, HBTU, HATU, TBTU in the presence of HoBt or HoAt).

The activating reagents used in the solid phase synthesis of the peptides are well known in the peptide art. Examples of suitable activating reagents are carbodiimides, such as N,N'-diisopropylcarbodiimide and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide. Other activating reagents and their use in peptide coupling are described by Schroder & Lubke (1965) and Kapoor (1970).

Each protected amino acid or amino acid sequence is introduced into the solid-phase reactor in about a twofold or more excess, and the coupling may be carried out in a medium of dimethylformamide (DMF):CH_2Cl_2 (1:1) or in DMF or CH_2Cl_2 alone. In cases where intermediate coupling occurs, the coupling procedure is repeated before removal of the α-amino protecting group prior to the coupling of the next amino acid. The success of the coupling reaction at each stage of the synthesis, if performed manually, is preferably monitored by the ninhydrin reaction, as described by Kaiser et al. (1970). Coupling reactions can be performed automatically, as on a Beckman 990 automatic synthesizer, using a program such as that reported in Rivier et al. (1978).

After the desired amino acid sequence has been completed, the intermediate peptide can be removed from the resin support by treatment with a reagent, such as liquid hydrogen fluoride or TFA (if using Fmoc chemistry), which not only cleaves the peptide from the resin but also cleaves all remaining side chain protecting groups and also the α-amino protecting group at the N-terminus if it was not previously removed to obtain the peptide in the form of the free acid. If Met is present in the sequence, the Boc protecting group is preferably first removed using trifluoroacetic acid (TFA)/ethanedithiol prior to cleaving the peptide from the resin with HF to eliminate potential S-alkylation. When using hydrogen fluoride or TFA for cleaving, one or more scavengers such as anisole, cresol, dimethyl sulfide and methylethyl sulfide are included in the reaction vessel.

Cyclization of the linear peptide is preferably affected, as opposed to cyclizing the peptide while a part of the peptido-resin, to create bonds between Cys residues. To effect such a disulfide cyclizing linkage, fully protected peptide can be cleaved from a hydroxymethylated resin or a

chloromethylated resin support by ammonolysis, as is well known in the art, to yield the fully protected amide intermediate, which is thereafter suitably cyclized and deprotected. Alternatively, deprotection, as well as cleavage of the peptide from the above resins or a benzhydrylamine (BHA) resin or a methylbenzhydrylamine (MBHA), can take place at 0°C with hydrofluoric acid (HF) or TFA, followed by oxidation as described above.

The peptides are also synthesized using an automatic synthesizer. Amino acids are sequentially coupled to an MBHA Rink resin (typically 100 mg of resin) beginning at the C-terminus using an Advanced Chemtech 357 Automatic Peptide Synthesizer. Couplings are carried out using 1,3-diisopropylcarbodimide in N-methylpyrrolidinone (NMP) or by 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and diethylisopro- pylethylamine (DIEA). The FMOC protecting group is removed by treatment with a 20% solution of piperidine in dimethylformamide(DMF). Resins are subsequently washed with DMF (twice), followed by methanol and NMP.

On the basis of the amino acid sequence of Mar1, oligonucleotide primers were synthesized and used in 5' and 3' RACE (rapid amplification of cDNA ends) procedures to isolate the gene encoding the Mar 1 precursor protein. Alternatively, the DNA to be probed is DNA which is isolated and cloned in accordance with conventional techniques using general procedures well known in the art, such as described in Olivera et al. (1996). One example of a suitable degenerate primers are CAGGATCCAA(T/C)GGIGT(C/G/T)TG(T/C)TG(T/C)GG (SEQ ID NO:5) for 3" RACE and CTGGATCCGG(G/A)TG(A/G)CA(C/A/G)A(A/G)(C/T)TT(A/G)TAICC SEQ ID NO:6) for 5" RACE. As is common with conotoxin peptides, the identified DNAs coding for Mar1 codes for a precursor peptide which is translationally modified to yield the Mar1 peptide.

Additional ap-conotoxin peptides are identified by cloning by reverse transcription-polymerase chain reaction (RT-PCR) from cone snail venom duct mRNA. The PCR primers are based on the DNA sequences coding for the precursor peptides of Mar1 and Mar2. RT-PCR of venom duct mRNA produces a product of about 250-300 nucleotides in *Conus* species that express ap-conotoxin genes. The PCR product is then cloned into a plasmid vector and individual clones are sequenced to determine the sequence of various ap-conotoxin genes. Alternatively, cDNA libraries are prepared from *Conus* venom duct using conventional techniques. DNA from single clones is amplified by conventional techniques using primers which correspond approximately to the M13 universal priming site and the M13 reverse universal priming site. Clones having a size

of approximately 250 nucleotides are sequenced and screened for similarity in sequence to Mar1 and Mar2. In this manner, ap-conotoxins are cloned from many *Conus* species.

Muteins, analogs or active fragments, of the foregoing ap-conotoxin peptides are also contemplated here. See, e.g., Hammerland et al (1992). Derivative muteins, analogs or active fragments of the conotoxin peptides may be synthesized according to known techniques, including conservative amino acid substitutions, such as outlined in U.S. Patents No. 5,545,723 (see particularly col. 2, line 50 to col. 3, line 8); 5,534,615 (see particularly col. 19, line 45 to col. 22, line 33); and 5,364,769 (see particularly col. 4, line 55 to col. 7, line 26), each incorporated herein by reference.

Pharmaceutical compositions containing a compound of the present invention or its pharmaceutically acceptable salts as the active ingredient can be prepared according to conventional pharmaceutical compounding techniques. See, for example, *Remington's Pharmaceutical Sciences*, 18th Ed. (1990, Mack Publishing Co., Easton, PA). Typically, an analgesic amount of the active ingredient will be admixed with a pharmaceutically acceptable carrier. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., intravenous, oral or parenteral. The compositions may further contain antioxidizing agents, stabilizing agents, preservatives and the like.

For oral administration, the compounds can be formulated into solid or liquid preparations such as capsules, pills, tablets, lozenges, melts, powders, suspensions or emulsions. In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents, suspending agents, and the like in the case of oral liquid preparations (such as, for example, suspensions, elixirs and solutions); or carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations (such as, for example, powders, capsules and tablets). Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be sugar-coated or enteric-coated by standard techniques. The active agent can be encapsulated to make it stable to passage through the gastrointestinal tract while at the same time allowing for passage across the blood brain barrier. See for example, WO 96/11698.

For parenteral administration, the compound may be dissolved in a pharmaceutical carrier and administered as either a solution or a suspension. Illustrative of suitable carriers are water,

saline, dextrose solutions, fructose solutions, ethanol, or oils of animal, vegetative or synthetic origin. The carrier may also contain other ingredients, for example, preservatives, suspending agents, solubilizing agents, buffers and the like. When the compounds are being administered intrathecally, they may also be dissolved in cerebrospinal fluid.

Administration of the active agent according to this invention may be achieved using any suitable delivery means, including:

(a) pump (see, e.g., Annals of Pharmacotherapy, 27:912 (1993); Cancer, 41:1270 (1993); Cancer Research, 44:1698 (1984));

(b), microencapsulation (see, e.g., U.S. Pat. Nos. 4,352,883; 4,353,888; and 5,084,350);

(c) continuous release polymer implants (see, e.g., U.S. Pat. No. 4,883,666);

(d) macroencapsulation (see, e.g., U.S. Pat. Nos. 5,284,761, 5,158,881, 4,976,859 and 4,968,733 and published PCT patent applications WO92/19195, WO 95/05452);

(e) naked or unencapsulated cell grafts to the CNS (see, e.g., U.S. Pat. Nos. 5,082,670 and 5,618,531);

(f) injection, either subcutaneously, intravenously, intra-arterially, intramuscularly, or to other suitable site; or

(g) oral administration, in capsule, liquid, tablet, pill, or prolonged release formulation.

In one embodiment of this invention, an active agent is delivered directly into the CNS, preferably to the brain ventricles, brain parenchyma, the intrathecal space or other suitable CNS location, most preferably intrathecally.

Alternatively, targeting therapies may be used to deliver the active agent more specifically to certain types of cells, by the use of targeting systems such as antibodies or cell-specific ligands. Targeting may be desirable for a variety of reasons, e.g. if the agent is unacceptably toxic, if it would otherwise require too high a dosage, or if it would not otherwise be able to enter target cells.

The active agents, which are peptides, can also be administered in a cell based delivery system in which a DNA sequence encoding an active agent is introduced into cells designed for implantation in the body of the patient, especially in the spinal cord region. Suitable delivery systems are described in U.S. Patent No. 5,550,050 and published PCT Application Nos. WO 92/19195, WO 94/25503, WO 95/01203, WO 95/05452, WO 96/02286, WO 96/02646, WO 96/40871, WO 96/40959 and WO 97/12635. Suitable DNA sequences can be prepared synthetically for each active agent on the basis of the developed sequences and the known genetic code.

The active agent is preferably administered in an therapeutically effective amount. The actual amount administered, and the rate and time-course of administration, will depend on the nature and severity of the condition being treated. Prescription of treatment, e.g. decisions on dosage, timing, etc., is within the responsibility of general practitioners or spealists, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of techniques and protocols can be found in *Remington's Parmaceutical Sciences*. Typically the conopeptides of the present invention exhibit their effect at a dosage range from about 0.001 mg/kg to about 250 mg/kg, preferably from about 0.05 mg/kg to about 100 mg/kg of the active ingredient, more preferably from a bout 0.1 mg/kg to about 75 mg/kg, and most preferably from about 1.0 mg/kg to about 50 mg/kg. A suitable dose can be administered in multiple sub-doses per day. Typically, a dose or sub-dose may contain from about 0.1 mg to about 500 mg of the active ingredient per unit dosage form. A more preferred dosage will contain from about 0.5 mg to about 100 mg of active ingredient per unit dosage form. Dosages are generally initiated at lower levels and increased until desired effects are achieved.

EXAMPLES

The present invention is described by reference to the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Isolation of ap-Conotoxins

Crude venom was extracted from *Conus marmoreus* venom ducts (Cruz et al.. 1976), and lyophilized. The crude, lyophilized venom was extracted with 1.1% acetic acid, and the crude extract was size fractionated using a Sephadex G-25 column. Further purfication was accomplished using reverse phase HPLC using either 0.1% TFA or 0.05% HFBA (heptafluorobutyric acid) with an acetonitrile gradient. Bioactivity was monitored by means of i.c.v. injection into mice. Once purified, the disulfide bonds were reduced and Cys residues carboxymethlylated. The peptides were then chemically sequenced using standard methods.

In accordance with this method, peptides Mar1 and Mar2 having the amino acid sequences described above were obtained.

EXAMPLE 2

Synthesis of Conopeptides

The synthesis of conopeptides, either the mature toxins or the precursor peptides, are separately performed using conventional protection chemistry as described by Cartier et al. (1996). Briefly, the linear chains are built on Rink amide resin by Fmoc procedures with 2-(1H-benzotriol-1-yl)-1,1,3,3,-tetramethyluronium tetrafluoroborated coupling using an ABI model 430A peptide sythesizer with amino acid derivatives purchased from Bachem (Torrence CA). Orthogonal protection is used on cysteines using the stable Cys(S-acetamidomethyl) on one pair of cysteines and the acid-labile Cys(S-trityl) on the other pair of cysteines. After removal of the terminal Fmoc protecting group and cleavage of the peptides from the resins, the released peptides are precipitated by filtering the reaction mixture into -10°C methyl t-butyl ether, which removed the protecting groups except on stably protected cysteines. The peptides are dissolved in 0.1% TFA and 60% acetonitrile and purified by RPLC on a Vydac C_{18} preparative column (22 x 250 mm) and eluted at a flow rate of 20 mL/min with a gradient of acetonitrile in 0.1% TFA.

The disulfide bridges in the conopeptides are formed as described in Cartier et al. (1996). Briefly, the disulfide bridges between now unprotected cysteines are formed by air oxidation which is judged to be complete by analytical RPLC. The monocyclic peptides are purified by RPLC on a Vydac C_{18} prepartive column (22 x 250 mm) and eluted with a gradient of acetonitrile in 0.1% TFA. Removal of S-acetamidomethyl groups and closure of the disulfide bridge between the remaining pair of cysteines is carried out simultaneously be iodine oxidation. The cyclic peptides are purified by RPLC on a Vydac C_{18} prepartive column (22 x 250 mm) and eluted with a gradient of acetonitrile in 0.1% TFA.

EXAMPLE 3

Isolation of cDNA Encoding ap-Conotoxins Mar 1 and Mar2

Based on the amino acid sequence of the Mar1 peptide, degenerate oligonucleotide primers were synthesized and used in 5' and 3' RACE (rapid amplification of cDNA ends) procedures to isolate the gene encoding the Mar1 precursor protein. For 3' RACE, the Mar1F primer was synthesized with the sequence CAGGATCCAA(T/C)GGIGT(C/G/T)TG(T/C)TG(T/C)GG (SEQ ID NO:5) corresponding to the amino acids NGVCCG (residues 1-6 of SEQ ID NO:2) of the Mar 1 conotoxin. For 5' RACE, the Mar1R reverse primer was synthesized with the sequence CTGGATCCGG(G/A)TG(A/G)CA(C/A/G)A(A/G)(C/T)TT(A/G)TAICC (SEQ ID NO:6)

corresponding to the amino acids GYKLCHP (residues 6-12 of SEQ ID NO:2) of the Mar 1 conotoxin. Each of these oligonucleotides includes a synthetic recognition site for the restriction enzyme Bam HI at the 5' end to facilitate cloning of the PCR products. *Conus marmoreus* mRNA was isolated and used to synthesize cDNA with adapter sequences appended to the 5' and 3' termini. The adapter sequences contain a region complementary to a universal amplification primer (Lib-U primer; AAGCTCGAGTAACAACGCAGAGT (SEQ ID NO:7)). The Lib-U primer contains a Xho I site to facilitate cloning of the PCR products. 3' RACE amplification of the *C. marmoreus* cDNA with the Mar1F and Lib-U primers generated a specific 620 bp PCR product, and 5' RACE with the Mar1R and Lib-U primers generated a 310 bp PCR product. Each of these PCR products was directionally cloned into the Bam HI and Xho I sites of the plasmid vector pBluescript II SK-. Plasmid clones containing inserts of the appropriate size were identified and DNA sequences were determined for several of the 5' RACE and 3' RACE clones. All of the 5' RACE and 3' RACE clones corresponded to the Mar 1 sequence. The Mar1F and Mar1R primers were designed to generate overlapping cDNA fragments, and by aligning the 5' RACE and 3' RACE sequences the complete Mar1 gene sequence was deduced.

The Mar1 cDNA sequence is 790 bp, followed by a poly A tail at the 3' end. The first open reading frame encountered from the 5' end of the cDNA initiates from a start codon at base pair 82, and encodes a protein of 61 amino acids. The Mar 1 conotoxin sequence resides at the C-terminus of this precursor protein, and is immediately preceded by an basic arginine residue. The first 24 amino acids of the precursor protein comprise a highly hydrophobic signal sequence. Each of these features is characteristic of conotoxin precursor protein structure. Following the stop codon, there is 522 bp of 3' untranslated region sequence. The DNA sequence of the signal sequence region and the 3' untranslated region can be used to design PCR primers to isolate conotoxin genes related to this novel Mar1 peptide from other *Conus* species. The Mar1 coding sequence (SEQ ID NO:8) and the Mar1 propeptide sequence (SEQ ID NO:9) are set forth in Table 1.

TABLE 1

DNA Sequence (SEQ ID NO:8) and Protein Sequence (SEQ ID NO:9) of Mar1

```
ggcgaataca cctggcaggt actcaacgaa cttcaggaca cattcttttc acctggacac

tggaaactga caacaggcag a atg cgc tgt ctc cca gtc ttg atc att ctt
                        Met Arg Cys Leu Pro Val Leu Ile Ile Leu

ctg ctg ctg act gca tct gca cct ggc gtt gtt gtc cta ccg aag acc
Leu Leu Leu Thr Ala Ser Ala Pro Gly Val Val Val Leu Pro Lys Thr
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gaa gat gat gtg ccc atg tca tct gtc tac ggt aat gga aag agt atc
Glu Asp Asp Val Pro Met Ser Ser Val Tyr Gly Asn Gly Lys Ser Ile

cta cga gga att ctg agg aac ggt gtt tgc tgt ggc tat aag ttg tgc
Leu Arg Gly Ile Leu Arg Asn Gly Val Cys Cys Gly Tyr Lys Leu Cys

cat cca tgt taaccagcat gaagggaaat gactttggat gagacccctg
His Pro Cys

cgaactgtcc ctggatgtga aatttggaaa gcagactgtt cctttcgcac gtattcgtgg

aatttcgaat ggtcgtaaac aacacgctgc cacttgcagg ctactatctc tctgtccttt

catctgtgga aatggatgat ctaacaactg aaatatcaga aatttttcaa tggctataca

ctatgaccat gtagtcagta attatatcat ttggaccttt tgaaatattt ttcaatatgt

aaagtttttg caccctggaa aggtcttttg gagttaaata ttttagtatg ttatgttttg

catacaagtt atagaatgct gtctttcttt ttgttcccac atcaatggtg ggggcagaaa

ttatttgttt tggtcaatgt aattatgacc tgcatttagt gctatagtga ttgcattttc

agcgtggaat gtttaatctg caaacagaaa gtggttgatc gactaataaa gatttgcatg

gcacaaaaaa aaaaaaaaaa a

EXAMPLE 4

Isolation of DNA Encoding ap-Conotoxins

Additional ap-conotoxin peptides are identified by cloning by reverse transcription-polymerase chain reaction (RT-PCR) from cone snail venom duct mRNA. The PCR primers are based on the DNA sequences coding for the precursor peptides of Mar1 and Mar2. RT-PCR of venom duct mRNA produces a product of about 250 nucleotides in *Conus* species that express ap-conotoxin genes. The PCR product is then cloned into a plasmid vector and individual clones are sequenced to determine the sequence of various ap-conotoxin genes. Alternatively, cDNA libraries are prepared from *Conus* venom duct using conventional techniques. DNA from single clones is amplified by conventional techniques using primers which correspond approximately to the M13 universal priming site and the M13 reverse universal priming site. Clones having a size of approximately 250-300 nucleotides are sequenced and screened for similarity in sequence to Mar1 and Mar2. In this manner, ap-conotoxins are cloned from many *Conus* species.

EXAMPLE 5

Analgesic Activity of Mar1 and Mar2

Intrathecal (it) drug injections were performed as described by Hylden et al. (1980). Mar1 (2.5 nmol), Mar2 (2.5 nmol) or water vehicle was administered to CF-1 mice (five mice per group)

in a volume of 5 μl. Twenty minutes after injection, the body temperature of each animal was determined. Thirty minutes after injection, each animal was placed on a 55° C hotplate. The latency to the first response (flinch), a spinally mediated behavioral response, and the first hindlimb lick, a centrally organized motor response to acute pain, were recorded. Mice were removed from the hotplate after 60 seconds if no response was observed. Forty-five minutes after injection, motor function for each mouse was tested by determining the latency to first fall from an accelerating rotarod. The results of these experiments are shown in Table 2. This data demonstrate that Mar1 and Mar2 have potent analgesic properties

TABLE 2

Analgesic Activity of Mar1 and Mar2

Assay	Vehicle	Mar1	Mar2
Body Temp (°C)	34.3 ± 0.3	34.2 ± 0.2	33.7 ± 0.7
Hot Plate			
First Response (s)	4.1 ± 0.3	4.0 ± 0.8	9.1 ± 2.0*
First Lick (s)	9.2 ± 0.7	31.7 ± 7.9	34.2 ± 8.5*
Accelerating Rota-Rod (s)	200 ± 50	144 ± 21	158 ± 40

mean ± S.E.M.; * $p < 0.05$ Dunnett's multiple comparison test.

EXAMPLE 6

Analgesic Activity of Mar1 and Mar2

Analgesic activity of Mar1 and Mar 2 was also tested in a persistent pain models as follows.

Persistent pain (formalin test). Intrathecal (it) drug injections were performed as described by Hylden at al (1980). Mar1, Mar2 or vehicle was administered in a volume of 5 μl. Fifteen minutes after the it injection, the right hindpaw was injected with 20 μl of 5% formalin. Animals were placed in clear plexiglass cylinders backed by mirrors to facilitate observation. Animals were closely observed for 2 minutes per 5 minute period, and the amount of time the animal spent licking the injected paw was recorded in this manner for a total of 45-50 minutes. Results were expressed as licking time in seconds per five minutes. At the end of the experiment, all animals were placed on an accelerating rotorod and the latency to first fall was recorded. Both Mar1 and Mar2 were found to be active in this model which is predictive of efficacy for treating neuropathic pain.

EXAMPLE 7

Analgesic Activity of Marl and Mar2

Analgesic activity of Marl and Mar 2 are also tested in further pain models as follows.

1. Acute pain (tail-flick). Marl, Mar2 or saline is administered intrathecally (i.t.) according to the method of Hylden et al. (1980) in a constant volume of 5 µl. Mice are gently wrapped in a towel with the tail exposed. At various time-points following the i.t. injection, the tail is dipped in a water bath maintained at 54° C. and the time to a vigorous tail withdrawal is recorded. If there is no withdrawal by 8 seconds, the tail is removed to avoid tissue damage.

2. Neuropathic pain. The partial sciatic nerve ligation model is used to assess the efficacy of Marl and Mar 2 in neuropathic pain. Nerve injury is produced according to the methods of Malmberg and Basbaum (1998). Animals are anesthetized with a ketamine/xylazine solution, the sciatic nerve is exposed and tightly ligated with 8-0 silk suture around 1/3 to ½ of the nerve. In sham-operated mice the nerve is exposed, but not ligated. Animals are allowed to recover for at least 1 week before testing is performed. On the testing day, mice are placed in plexiglass cylinders on a wire mesh frame and allowed to habituate for at least 60 minutes. Mechanical allodynia is assessed with calibrated von Frey filaments using the up-down method as described by Chaplan et al. (1994), and the 50% withdrawal threshold is calculated. Animals that did not respond to any of the filaments in the series are assigned a maximal value of 3.6 grams, which is the filament that typically lifted the hindlimb without bending, and corresponds to approximately 1/10 the animal's body weight.

The data obtained demonstrate that Mar 1 and Mar2 have potent analgesic properties in three commonly used models of pain: acute, persistent/inflammatory and neuropathic pain models. Marl and Mar2 administered intrathecally reduced the response latency in the tail flick model of acute pain, and was effective in the low nanomole range. Marl and Mar2 also showed analgesic activity in a model of neuropathic pain.

It will be appreciated that the methods and compositions of the instant invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the artisan that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

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U.S. Patent No. 3,972,859.

U.S. Patent No. 3,842,067.

U.S. Patent No. 3,862,925.

U.S. Patent No. 5,550,050.

PCT Published Application WO 92/19195.

PCT Published Application WO 94/25503.

PCT Published Application WO 95/01203.

PCT Published Application WO 95/05452.

PCT Published Application WO 96/02286.

PCT Published Application WO 96/02646.

PCT Published Application WO 96/11698.

PCT Published Application WO 96/40871.

PCT Published Application WO 96/40959.

PCT Published Application WO 97/12635.

WHAT IS CLAIMED IS:

1. A substantially pure ap-conotoxin peptide having the generic formula I: Xaa_1-Gly-Xaa_2-Cys-Cys-Gly-Xaa_3-Xaa_4-Xaa_5-Cys-Xaa_6-Xaa_7-Cys (SEQ ID NO:1), wherein Xaa_1 is Asn or des-Xaa_1; Xaa_2 is Val or Ile; Xaa_3 is Val, Tyr, mono-halo-Tyr, di-halo-Tyr, O-sulpho-Tyr, O-phospho-Tyr, nitro-Tyr; Xaa_4 is Set, Thr, Lys, N-methy-Lys, N,N-dimethyl-Lys, N,N,N-trimethyl-Lys, Arg, homoarginine or ornithine; Xaa_5 is Leu or Phe; Xaa_6 is His, halo-His, Tyr, mono-halo-Tyr, di-halo-Tyr, O-sulpho-Tyr, O-phospho-Tyr, nitro-Tyr; Xaa_7 is Pro or hyroxy-Pro; and the C-terminus contains a free carboxyl group or an amide group.

2. The substantially pure ap-conotoxin peptide of claim 1 seleceted from the group consisting of:

Asn-Gly-Val-Cys-Cys-Gly-Xaa_1-Xaa_2-Leu-Cys-His-Xaa_3-Cys (SEQ ID NO:2);
Gly-Val-Cys-Cys-Gly-Xaa_1-Xaa_2-Leu-Cys-His-Xaa_3-Cys (SEQ ID NO:3) and
Gly-Ile-Cys-Cys-Gly-Val-Ser-Phe-Cys-Xaa_1-Xaa_3-Cys (SEQ ID NO:4),

wherein Xaa_1 is Tyr, mono-halo-Tyr, di-halo-Tyr, O-sulpho-Tyr, O-phospho-Tyr, nitro-Tyr; Xaa_2 is Lys, N-methy-Lys, N,N-dimethyl-Lys or N,N,N-trimethyl-Lys; Xaa_3 is Pro or hydroxy-Pro, preferably hydroxy-Pro; and the C-terminus contains a carboxyl or amide group.

3. The substanially pure ap-conotoxin peptide of claim 2, wherein Xaa_1 is Tyr.

4. The substanially pure α-conotoxin peptide of claim 3, wherein Xaa_2 is Lys.

5. The substanially pure ap-conotoxin peptide of claim 2, wherein Xaa_2 is Lys.

6. The substanially pure ap-conotoxin peptide of claim 2, wherein Xaa_3 is hydroxy-Pro.

7. The substanially pure ap-conotoxin peptide of claim 3, wherein Xaa_3 is hydroxy-Pro.

8. The substanially pure ap-conotoxin peptide of claim 4, wherein Xaa_3 is hydroxy-Pro.

9. The substanially pure ap-conotoxin peptide of claim 5, wherein Xaa_3 is hydroxy-Pro.

10. The substanially pure ap-conotoxin peptide of claim 2, wherein halo is iodine.

11. The substanially pure ap-conotoxin peptide of claim 10, wherein Xaa_3 is mono-iodo-Tyr.

12. The substanially pure ap-conotoxin peptide of claim 10, wherein Xaa_3 is di-iodo-Tyr.

13. The substantially pure ap-conotoxin peptide of claim 2, wherein said peptide has the sequence set forth in SEQ ID NO:2, wherein Xaa_1 is Tyr, Xaa_2 is Lys and Xaa_3 is hydroxy-Pro.

14. The substantially pure ap-conotoxin peptide of claim 2, wherein said peptide has the sequence set forth in SEQ ID NO:3, wherein Xaa_1 is Tyr, Xaa_2 is Lys and Xaa_3 is hydroxy-Pro.

15. The substantially pure ap-conotoxin peptide of claim 2, wherein said peptide has the sequence set forth in SEQ ID NO:4, wherein Xaa_1 is Tyr and Xaa_3 is hydroxy-Pro.

16. A method for inducing analgesia in a mammal which comprises administering a therapeutically effective amount of a ap-conotoxin peptide of claim 1.

17. The method of claim 16, wherein administration is intrathecal.

18. The method of claim 16, wherein administration is intracerebroventricular.

19. The method of claim 16, wherein administration is parenteral.

20. The method of claim 16 wherein the amount of ap-conotoxin peptided administered is between about 0.001 mg/kg to about 250 mg/kg.

21. An isolated nucleic acid comprising a nucleotide sequence coding for Marl propeptide comprising the amino acid sequence set forth in SEQ ID NO:9.

22. The isolated nucleic acid of claim 21 comprising the nucleotide sequence set forth in SEQ ID NO:8.

23. An isolated Marl propeptide comprising the amino acid sequence set forth in SEQ ID NO:9.

SEQUENCE LISTING

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1 5 10

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     60

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/01978

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :C07K 5/00; C12N 15/11; A61K 38/00
US CL : 530/300; 536/23.1; 514/2-21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/300; 536/23.1; 514/2-21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,432,155 A (OLIVERA et al.) 11 July 1995 (11.07.95), see entire document.	1-23
A	US 5,633,347 A (OLIVERA et al.) 27 May 1997 (27.05.97), see entire document.	1-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 23 APRIL 2000	Date of mailing of the international search report 06 JUN 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JAYAVANTHI SATISH Telephone No. (703) 306-9047

Form PCT/ISA/210 (second sheet) (July 1998)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/01978

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

BIOSIS CAPLUS MEDLINE JAPIO EMBASE

ap-conotoxin peptide, DNA encoding, Mar 1 propeptide, analgesic activity